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14. ABSTRACT

To metastasize, cancer cells have to break through basement membrane (BM). Laminin-5 is one of the most abundant BM proteins. It consists of three chains α3, β3 and γ2. DIII domain, a functional EGFR ligand will be released from Ln-5 γ2 chain by MMPs processing. It has been suggested by our lab that DIII domain may facilitate cancer progression by preventing anoikis. There are paradoxical data in regard of the role of Ln-5 in cancer progression. For example, both the increased and decreased expression levels of Ln-5 subchains are reported in the literature. The fact that γ2 chain exists in two different forms (as a secreted monomer, or as a part of the Ln-5 heterotrimer) leads us to hypothesize that those two forms may play different roles in cancer progression. In this proposal we will determine if the expression of Ln-5 γ2 monomer is positively correlated with breast cancer cell line tumorigenecity. In addition, we will determine the role of Ln-5 γ2 chain in cancer progression when it is in the context of Ln-5 heterotrimer.

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Introduction:

To metastasize, cancer cells have to break through the basement membrane. Ln-5 is one of the basement membrane proteins, consisting of three chains α3, β3 and γ2. Ln-5 γ2 chain contains DIII domain, a functional EGFR ligand, which can be released by MMP processing. It has been suggested by our lab that DIII domain may facilitate cancer progression by preventing anoikis.

What we noticed is that there are paradoxical data in regard of the role of Ln-5 in cancer progression. For example, both the increased and decreased expression levels of Ln-5 subchains are reported in the literature.

The fact that γ2 chain exists in two different forms (as a secreted monomer, or as a part of the Ln-5 heterotrimer) leads us to hypothesize that those two forms may play different roles in cancer progression.

Therefore, the original aims are expanded and modified as the following two: A). To determine if the expression of Ln-5 γ2 monomer is positively correlated with breast cancer cell line tumorigenecity. B). To determine the role of Ln-5 γ2 chain in cancer progression when it is in the context of Ln-5 heterotrimer.

Body:

Accomplishments

A. To determine if the expression of Ln-5 γ2 monomer is correlated with the tumorigenicity of breast cancer cell lines. (This work is done in collaboration with Cherise Guess , graduate student in our lab)

To detect whether γ2 chain exists as a monomer, we decided to check the ratio between Ln-5 β3 and γ2 (β3/γ2 ratio)[1, 2]. The lower the ratio is, the more likely it is for γ2 monomers to be secreted . For the preliminary screening in cell lines, we used Realtime-PCR (RT-PCR). So far, we compared β3/γ2 between human breast gland cell lines MCF10A (non tumorigenic) and MCF10A-CA1a (tumorigenic). Our preliminary analysis reveals that the β3/γ2 ratio of the CA1a cells is lower than the β3/γ2 ratio of the MCF10A cells, which indicates that MCF10ACA1a may produce more γ2 monomer compared with MCF10A

B. To determine the role of Ln-5 γ2 chain in cancer progression in the context of Ln-5 heterotrimer.

For this task, we proposed to knock down Ln-5 γ2 chain in MCF10A cells, which synthesize a large amount of Ln-5 heterotrimer. We are still working on generating Ln-5 γ2 knockdown MCF10A. However, we did make significant progress in understanding the question by using 804G cells. 804G is a rat bladder carcinoma cell line, but it shares two important and unique properties with MCF10A, rarely seen in other cell lines: it secretes a large amount of Ln-5 heterotrimer and assembles hemidesmosomes in vitro. Ln-5 γ2 is 75% knocked down in 804G cells by small hairpin RNA (shRNA) as shown by western blotting in Fig1. The correspondent scrambled shRNA were used as control. (shRNA sequences are in fig1.) Two stable cell lines were

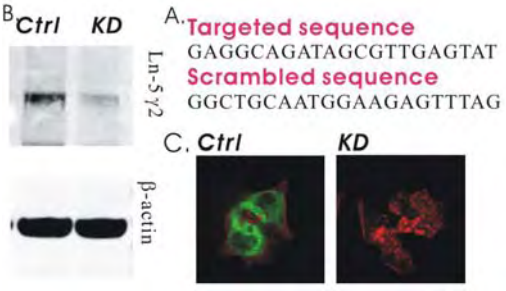

Fig. 1. shRNA knockdown of Rat γ2 chain in 804G cells. A. The targeted and the corresponding scrambled sequences . **B**. Western blot analysis by using Ln-5 antibody 2778. **C.** 804G-γ2-kd and 804G-γ2-ctrl were stained by Ln-5 antibody 2778 (green) and phalloindin(red)

generated, which are 804G-γ2-kd and 804G-γ2-ctrl.

Comparison of growth rates The growth rate of 804G cells is slightly decreased by Ln-5 γ2 knockdown. Initially, $2X10^4$ cells were seeded in 6-well plastic culture plate in 10% FBS supplemented DMEM. Cells were counted under microscope every 24 hours after trypan blue staining. The doubling times for ctrl and kd cells are 22hr and 24hr, respectively. 804G-γ2-kd cells grow faster than 804G-γ2-ctrl cells.

Migration Random cell migration was analyzed by Metamorph-based time-lapse microscopy. Cells were plated on 35mm plastic cell culture dishes three hours before taking the movie. Movies were analyzed by with Metamorph. Fig.2 shows the average velocity of cell migration in three hours. As shown in Fig.2., 804G-γ2-ctrl cells move two times faster than 804G γ2-ctrl.

Invasion Matrigel coated transwells were used to evaluate the invasiveness of 804G-γ2-ctrl and 804G-γ2-kd cells. The upper chamber was coated with a thin layer of Matrigel. $6X10^4$ cells in serum free DMEM were plated in the upper chamber, 600μl 10% FBS DMEM was used as chemoattractant in the lower chamber.

After 16hrs, cells on the lower face of filter were fixed and stained by crystal violet. Cell number was counted in five fields under light microscope. In this assay, 804G-γ2-kd cells were much less invasive than 804G-γ2-ctrl cells.

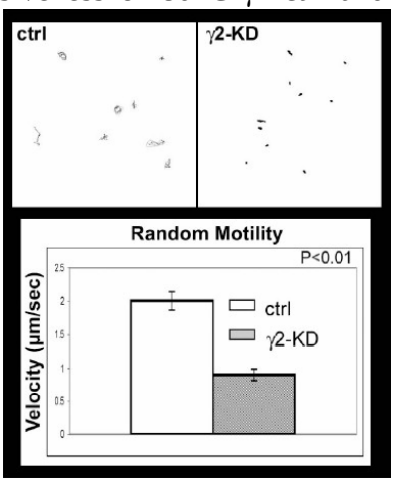

Fig.2. Ln-5 knockdown decreases the random migration speed of 804G cells. Top: tracks from representative movies of 804G-γ2-ctrl and 804G-γ2-kd cells. Bottom: mean +/- SE single cell velocity

804G-γ2-kd are dramatically more tumorigenic under the kidney capsule in SCID mice than 804G-γ2-ctrl

Instead of the CAM model we proposed to use in our proposal, we applied our 804G-γ2-ctrl and 804G-γ2-kd cells in the kidney capsule model (Fig.2 A). The advantages are 1) this model is well established at Vanderbilt by Dr. Simon Hayward, who pioneered this technique in Dr. Gerry Cunha laboratory (UCSF) and is now our collaborator; 2) this model allow us to detect local invasion as well as organ metastasis in one month, 3) the host of this model is the mouse which is closer to human than chick (CAM model).

To be able to track cells in vivo, 804G-γ2-kd and 804G-γ2-ctrl cells were engineered to express GFP-actin by retroviral infection. $5X10^5$ cells of each cell lines were resuspended in 50μl of neutralized type I rat tail collagen and incubated for 30min at 37 before medium was added. Cells in collagen gel were cultured at 37 overnight. They were then grafted beneath the renal capsule of adult male severe combined immunodeficient (SCID) mice [C.B.17/IcrHsd-scid (Harlan)]. Four weeks later, grafts were taken out from kidney and fixed by formalin overnight. Haematoxylin and Eosin Staining (H&E) was performed to check the histology. GFP staining was performed to confirm that those grafts were formed by the modified 804G cells. As shown in Fig. 3, 804G-γ2-ctrl cells only form a thin layer of white tumor like tissue on the kidney, while 804G-γ2-kd cells form a tumor, as big as the kidney.

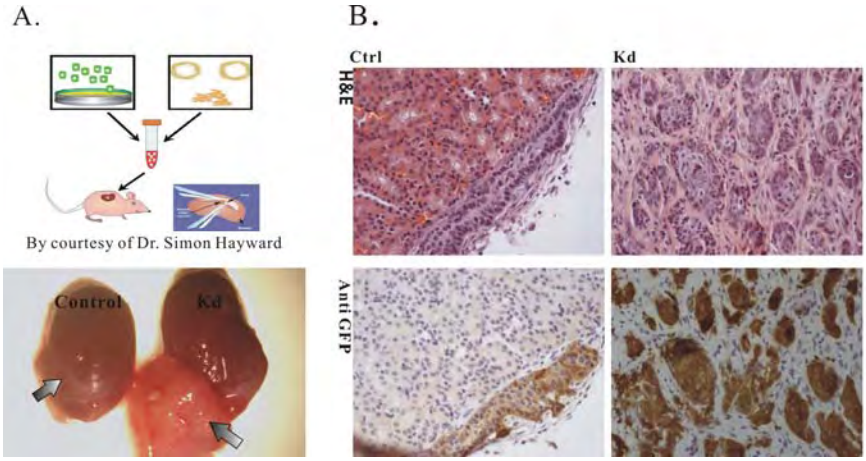

Fig.3 A. Top: Renal capsule model. Bottom: representative images of 804G-γ2-ctrl and 804G-γ2-kd tumors growing under the kidney capsule. The tumors are indicated by arrows. 804G-γ2-kd cells form a large tumor under kidney capsule while 804G-γ2-ctrl cells form a thin white layer under kidney.
B. Top: H&E staining to show the histology of tumors formed by kd and ctrl cells. Bottom: GFP staining to identify implanted 804G cells

Cell-cell junctions

The 804G-γ2-kd cells gain a fibroblast like morphology on culture plate, which is different from the 804G-γ-ctrl cells. Cell-cell adhesion in 804G-γ2-kd is disrupted obviously (Fig.4 top panel). E-cadherin (E-cad) staining was performed on the tumor sections (Fig.4 bottom panel). In 804G-γ2-ctrl graft, E-cad localizes to cell-cell junctions, whereas in 804G-γ2-kd graft E-cad localizes to the cytoplasma. Ln-5 γ2 staining suggests that E-cad might translocate into cytoplasm in the absence of Ln-5 heterotrimer.

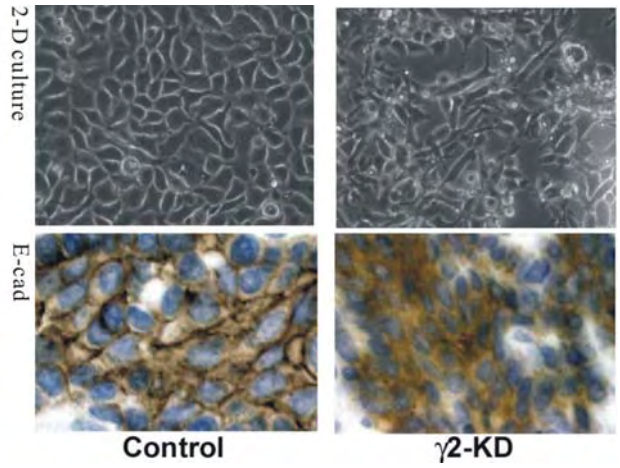

Fig.4.
Top: 804G-γ2-kd cells changed the morphology in 2-D culture.
Bottom: E-cadherin staining on tumor sections. E-cad localizes to cell-cell junctions in 804G-γ2-ctrl cells, but is diffusely cytoplasmic in 804G-γ2-kd cells.

Key research accomplishments

A. RT-PCR showed that MCF10ACA1a (tumorigenic) has more γ2 monomer than MCF10A (non tumorigenic).

B. Downregulation of Ln-5 γ2 by shRNA in 804G cells which synthesize Ln-5 heterotrimer leads makes 804G cells more tumorigenic. E-cad staining on tumor sections suggests that loss of γ2 chain in the context of Ln-5 heterotrimer leads to loss of E-cad at cell-cell junction.

Reportable outcomes

The work so far done in this project has been presented in the department seminar at Vanderblit University. Also, the poster was presented in the American Society for *M*atrix Biology Biennial National Meeting 2006.

Conclusions

Overexpression of Ln-5 γ2 chain had been associated with increased tumorigenesis, in breast as well as other cancers. Surprisingly, we have found that deletion of γ2 chain expression, instead, can dramatically upregulate tumorigenesis in *in vivo* models. However, there is an

important distinction to be made: if deletion of γ2 chain expression occurs in the context of a cell line that secretes Ln-5 heterotrimers, then such deletion results in lack of heterotrimer formation. In contrast, we hypothesize tha if deletion of γ2 chain occurs in the context of a cell that secretes γ2 monomer, than reduced tumorigenesis should be observed. In summary, we tentatively conclude that Ln-5 heterotrimers have a tumor suppressor role, γ2 chain monomer instead have a tumor promoting role.

Future direction

In the next year, we will continue our screening of Ln-5 γ2 monomer expression in MCF10A and derivatives by RT-PCR. We will knockdown γ2 in breast cancer cell lines which are expressing monomer. How does loss of γ2 monomer will affect tumorigenecity of breast cancer cell lines will be determined in vitro (soft agar assay) and in vivo (renal capsule mouse model).

For our task B, we will knockdown Ln-5 γ2 chain in MCF10A cells which express Ln-5 heterotrimer. The in vitro and in vivo assays used in 804G cells will be applied to MCF10A-γ2-kd and ctrl cells. We will also try to understand the underlying mechanism.

1. Nomizu, M., et al., *Assembly of synthetic laminin peptides into a triple-stranded coiled-coil structure.* J Biol Chem, 1994. **269**(48): p. 30386-92.
2. Matsui, C., et al., *The assembly of laminin-5 subunits.* J Biol Chem, 1995. **270**(40): p. 23496-503.